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A METHOD OF TRANSMITTING KNOWN NUMBERS OF TRYPANOSOMES WITH A NOTE ON THE NUMERIC RELATION OF TRYPANOSOMES TO INFECTION *

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This method for transmitting trypanosomes was evolved during chemotherapeutic researches in which several species of these parasites were used as the test organism, by reason of the necessity for having a fairly accurate, rapid, and simple means of infecting rats with known numbers of trypanosomes. As will be pointed out later in this paper, the time of true incubation of trypanosomes following infection by the intraperitoneal route, as well as to some extent the duration of the animal's life, are factors influenced by the numbers of trypanosomes injected; or, even more important, as will be discussed in a later paper, is the fact that the parasitropic effects of a drug or substance under study and the resistance of the animal to the drug's organotropic effects are apparently modified, to some extent at least, by whether the animal was infected with small or large numbers of trypanosomes. While it is necessary to use a parasite of sufficient aggressiveness and vitality to yield definite results and indicate any therapeutic action of the agent under study, yet it is possible to mask the finer grades of therapeutic effects by an overwhelming infection, or, on the other hand, lose or vitiate an experiment by infecting with too few parasites.

With these practical considerations in mind, I have worked out a method for transmitting approximately known numbers of trypanosomes, which has proved comparatively simple, fairly accurate, and very useful. By this method, it is possible to infect a large series of rats with any desired number of trypanosomes, in a sterile manner, and without sacrificing the seed animal. It is highly desirable, especially in chemotherapeutic researches, to infect the animals with a uniform number of parasites, not only the animals of a single series, but likewise those of different series, and this method has been found to fulfill this important requirement satisfactorily.

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Briefly, the method consists in securing a definite volume of blood in a blood corpuscle-counting pipet and mixing it with a definite amount of a diluting fluid that hemolyses the erythrocytes, and fixes and stains the trypanosomes. The trypanosomes are then counted by means of a Thoma-Zeiss counting chamber and the number in a cubic centimeter of undiluted blood calculated. With this data at hand, 0.1 c.c. of blood is drawn aseptically from the heart of the seed animal in 0.9 c.c. of a 10 percent solution of sodium citrate in normal salt solution. With this blood, dilutions are made in warm, sterile, normal salt solution until a cubic centimeter or a fraction thereof contains the required dilution and number of trypanosomes determined upon for the purpose of infecting a series of rats, or for any other purpose.

TECHNIC

Seed Animal.—For the purpose of making an accurate count, the seed animal should be one that has not been infected over three or four days Especially is this true of rats infected with Trypanosoma brucei and Trypanosoma equiperdum, as after this time the parasites in the peripheral blood may be very numerous and, what is more important, may be gathered in conglomerated masses which cannot be broken up in the mixing pipet and which render any attempts at counting absolutely worthless. With rats infected with Trypanosoma lewisi, older infections than those mentioned may be found satisfactory, as the number of parasites is not usually so great as found with the more pathogenic species, and conglomeration is not so likely to occur.

A preliminary examination of the blood is first made in the usual manner by snipping off the tip of the tail and quickly transferring a drop of the blood on a clean cover glass to a microscopic slide. The weight of the glass is allowed to spread the blood. This preparation is then examined by means of a No. 1/6″ objective and No. 4 eyepiece (Leitz), and the number of trypanosomes recorded according to the following scheme:

++++ = very large numbers; cannot be counted.
+++ = large numbers that may be roughly counted (not accurately).
++ = about 10-20 in a field.
+ = about 5-10 in a field.
Few = 1-2 in a field, or in every other field.

This preliminary examination is necessary because it indicates the blood pipet to use, as the red corpuscle pipet for a 1:200 dilution with ++++ and +++ rats, and the white corpuscle pipet for a 1:20 dilution with ++ and + rats. This method of recording the count of trypanosomes has been found useful as a routine procedure in the daily examination of large numbers of rats in our chemotherapeutic researches and with due care it yields a satisfactory record.

The Diluting Fluid.—After some experimentation a fluid has been adopted which serves the three-fold purpose of (1) fixing the trypanosomes and rendering them motionless, (2) quickly staining the trypanosomes so that they are readily seen and counted, and (3) removing the erythrocytes by hemolysis so that the parasites are readily seen in a clear field. It is easily prepared and comparatively stable, remaining clear, without sediment, for many weeks and months at ordinary room temperature.

This fluid is prepared as follows:

Formalin (40 percent)	2 c.c.
Glacial acetic acid	2 c.c.
Distilled water	96 c.c.

Mix and add 2 c.c. carbolfuchsin (Ziehl-Neelson); mix again and filter through paper. This quantity of fuchsin in the presence of formalin gives the fluid a clear, deep-purplish red color. Probably less fuchsin will prove satisfactory to others; more of the dye deepens the color of the fluid unnecessarily and may somewhat obscure the lines of the counting chamber. The formalin quickly destroys the motility of trypanosomes and keeps the fluid free of bacteria and fungi. The container should be well stoppered to prevent evaporation, and it may be advisable to refilter the fluid at occasional intervals.

The Counting of the Trypanosomes.—After the seed animal has been selected, a count is made of the trypanosomes. The tail is gently whipped back and forth for a minute or two to produce congestion; then, upon very gentle pressure, a large drop of blood appears where the tip has been snipped off. With the red corpuscle pipet, blood is carefully drawn to the mark 0.5 and the diluting fluid to the mark 101, the whole procedure being conducted with the care and exactness required for making an erythrocyte count. If the white corpuscle pipet is used, a large drop of blood is required and hence, before attempting to fill the pipet, a free flow of blood must be assured. With this pipet, blood is drawn to the mark 0.5 and the diluting fluid to the mark 11, as in the usual technic for counting leukocytes. As a general rule, the red blood corpuscle pipet is indicated and is more satisfactory than the white blood corpuscle pipet.

The filled pipet is now thoroughly shaken for at least one or two minutes; several drops are expelled, and then sufficient fluid is placed in the chamber and the cover glass accurately adjusted so that there is no fluid in the moat and Newton's color rings are seen. I never drop the cover glass on the chamber, but prefer to slide it quickly into place, firmly pressed to the slide. The slide is allowed to rest on a flat surface for at least ten minutes to permit the trypanosomes to sink down on the ruled off spaces.

By means of the No. 1/6″ objective and No. 4 or No. 2 eyepiece (Leitz) and a mechanical stage, all the trypanosomes in the ruled off space (400 small squares) of the Thoma ruling are counted. The trypanosomes are motionless, deeply stained a purplish red, and easily seen and counted. At times they are curled or placed edgewise, but with a clean diluting fluid, to avoid artefacts, and a little experience these forms are readily recognized. The red blood corpuscles have entirely disappeared, or appear as faint shadows. The leukocytes also are deeply stained, and a total leukocyte count may be made at the same time. A differential leukocytic count is also possible, at least as far as differentiating between the polynuclear and mononuclear leukocytes (Fig. 1).

In counting, it is well to start in the upper left hand corner and move across to the right, returning to the left, and so on, until the 400 small squares have been counted. If many trypanosomes are present, it is well to note on paper the number found in each of the four large squares across the ruled off space including the double lines; if few are present, the whole ruled off space may be counted in one figure. Trypanosomes placed half or more within the lines are included; otherwise, they are not included in the count. As a general rule, it is well to go over the slide twice; the totals should not differ by more than one or two trypanosomes and the counts of different persons should give the same tally and usually do after brief experience. These counts are more accurate than those ordinarily made of human erythrocytes and in every way as accurate as a leukocytic count made with equal care.

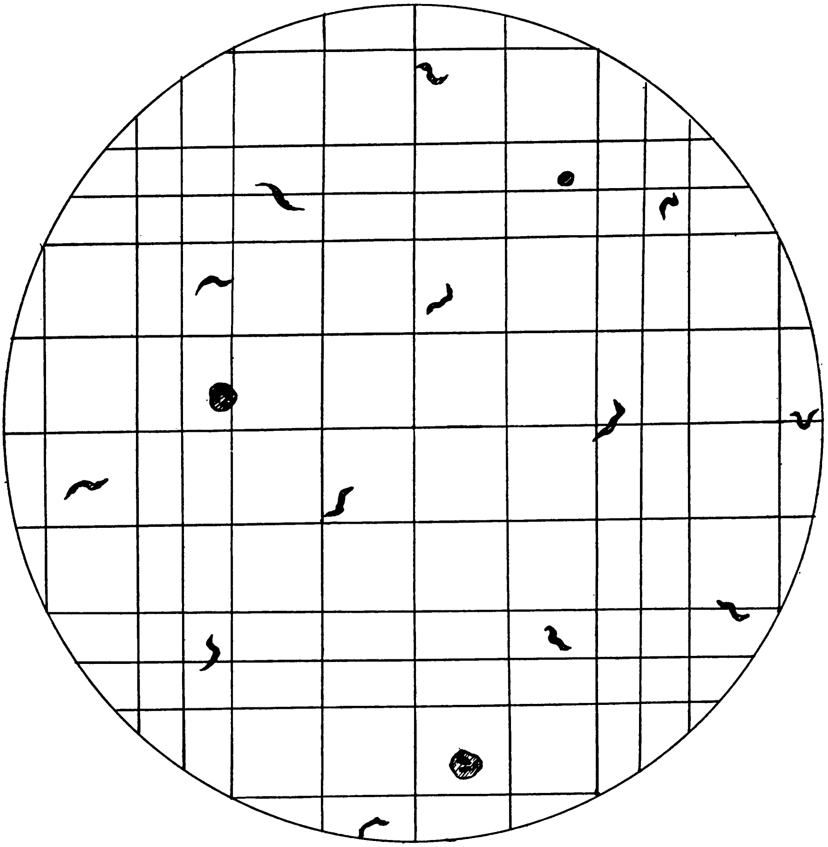

Fig. 1.—A section of the four hundred small squares of the Thoma ruling, showing trypanosomes in blood, diluted 1:200 (182,000,000 per cubic centimeter of undiluted blood), from a + + + + rat infected with Trypanosoma lewisi. X 288 (Leitz No. 6, eyepiece No. 2)

The Calculation of the Number of Trypanosomes in a Cubic Centimeter Undiluted Blood.—As each square contains 1/4000 cubic millimeter when the cover glass is properly adjusted, it is necessary to count the trypanosomes in 4,000 small squares to obtain the number contained in 1 cubic millimeter of the diluted blood. By counting the trypanosomes in 400 small squares and multiplying by 10, the approximate number in 4,000 small squares, or 1 cubic millimeter, is obtained. By multiplying this figure by the dilution as 200, if the red blood corpuscle pipet was used, or by 20 in case the white blood corpuscle pipet was used, the number in 1 cubic millimeter of undiluted blood is obtained. Multiplying this figure by 1,000 gives the approximate number of trypanosomes in a cubic centimeter of undiluted blood. An example briefly illustrates the method:

42	trypanosomes in ruled off area (400 small squares)
× 10	
420	trypanosomes in 4,000 small squares, or in one cubic millimeter of blood diluted 1:200
× 200	
84,000	trypanosomes in one cubic millimeter of undiluted blood
× 1,000	
84,000,000	trypanosomes in one cubic centimeter of undiluted blood

Briefly, by rule of thumb, count the trypanosomes in 400 small squares, multiply by 200, and add four ciphers. The result is the approximate number of trypanosomes in a cubic centimeter of undiluted blood.

If the white corpuscle pipet was used, the calculations are the same, except that the dilution is 1:20, as illustrated in another example:

93	trypanosomes in 400 small squares
× 10	
930	trypanosomes in 4,000 small squares, or one cubic millimeter of blood diluted 1:20
× 20	
18,600	trypanosomes in one cubic millimeter of undiluted blood
× 1,000	
18,600,000	trypanosomes in one cubic centimeter of undiluted blood

Or, by rule of thumb, count the trypanosomes in 400 small squares, multiply by 20, and add four ciphers.

A larger number of squares may be counted after a well known method for counting leukocytes; namely, by adjusting the microscope in such manner that the diameter of the field is just exactly eight small squares when the entire field contains fifty squares ($\pi r^2 = 3.1416 \times 16 = 50.26$); by counting the trypanosomes in twenty such fields, 1,000 small squares are covered, and multiplying this number by 80 gives the number of trypanosomes in 80,000 small squares, or one cubic millimeter of undiluted blood, and this figure multiplied by 1,000 gives the number of parasites in a cubic centimeter of undiluted blood. In the first method, 400 small squares are counted and in this method, 1,000 squares; comparative counts have been found to tally closely.

I have also tried the method of counting trypanosomes which consists in preparing a smear of the undiluted blood directly from the tail on a microscopic slide or on cover glasses, fixing with methyl alcohol, and staining with a suitable dye, as 1:10 carbolfuchsin, and then counting the trypanosomes and red blood corpuscles after the same manner in which Wright counts a bacterial vaccine. This method is relatively crude, yielding too variable results in different smears from the same rat prepared at the same time. The trypanosomes are frequently distributed in an irregular manner, and the number of erythrocytes in a cubic millimeter of normal rat blood is too valuable to adopt that figure as reliable which forms the basis of this method.

A question of considerable importance in this connection is whether counts of trypanosomes made with blood from the tail may be accepted as reliable and similar to counts made with blood from the heart, especially as blood for the purpose of transmitting the parasites is obtained from the heart of the seed animal.

I have made a series of counts of blood obtained from the tail, and from the right and left ventricles of the heart. The results of five of these comparative counts are shown in Table 1.

TABLE 1

COMPARATIVE COUNTS OF TRYPANOSOMES IN BLOOD FROM THE TAIL AND FROM THE HEART OF RATS

Rat	Infection	Direct Examination	Count of Blood from:		
			Tail	Right Ventricle	Left Ventricle
1	T. lewisi...............	++++	42,000,000	44,000,000	42,000,000
2	T. lewisi...............	++++	44,000,000	44,000,000	30,000,000
3	T. lewisi...............	++++	48,000,000	50,000,000	46,000,000
4	T. equiperdum.........	++++	1,400,000,000	1,360,000,000	1,200,000,000
5	T. lewisi...............	++++	40,000,000	41,000,000	40,000,000

As will be noted, the counts were found to run fairly parallel, especially in view of the large number of trypanosomes present and the chances of manipulative error. Frequently counts of blood obtained from the left ventricle showed slightly fewer trypanosomes than those of blood from the right ventricle and tail, but on the whole they were sufficiently close for the purpose of counting trypanosomes in the blood from the tail and using this figure as representing the number present in blood from the heart in the transmission of approximately known numbers of trypanosomes. It must be emphasized, however, that blood from the tail must flow easily and freely before counts are reliable; those from blood obtained after squeezing and massage of the tail are inaccurate and unreliable.

The Securing of Infected Blood for Transmissions.—Blood is obtained from the heart of the seed animal after the method I have described in a previous paper.[1] In this way, 0.1 c.c. of blood may be obtained in an accurate and sterile manner, the success of this method of transmitting known numbers of trypanosomes depending of course upon the operator's success in obtaining the blood accurately and quickly from the heart. There must be no more and no less blood, and the dilutions must be accurate.

This amount of blood is easily obtained from the living rat. If it cannot be drawn accurately, the animal should be killed, the thorax opened, and the blood drawn directly from the heart under the eye of the operator. With a little practice, however, one seldom fails to draw the blood from the living rat, and usually the operation does not appear to injure the animal.

It is necessary to use an accurate and well adjusted 1 c.c. syringe divided into tenth cubic centimeters. I prefer the Record syringe fitted with a No. 22 needle. A proper needle is very important; it must be sharp and sufficiently large to permit the easy flow of blood and yet not so large as unnecessarily to injure the heart.

[1] Jour. Infect. Dis., 1915, 16, p. 311.

(1) Warm a test tube containing 10 percent sodium citrate in normal salt solution in a Bunsen burner and draw up enough of this into the syringe to make, when air has been expelled, 0.4 c.c., including the needle. (The sodium citrate prevents coagulation of the blood under isotonic conditions.)

(2) The rat being held by an assistant, or fastened to an operating board, paint the left thorax with tincture of iodin and quickly insert the needle at about the point of maximal pulsation. By gentle suction on the piston, blood is made to flow into the syringe. If blood does not appear, re-insert the needle in an upward and inward direction without withdrawing it entirely. Draw blood accurately to the 0.5 c.c. mark (= 0.1 c.c. blood). Withdraw the needle and draw more of the warm citrate solution to the 1 c.c. mark. This gives a dilution of blood in the syringe equal to 1 in 10. Shake gently in order to mix the blood.

(3) Expel the contents of the syringe into a test tube containing 9 c.c. of warm salt solution and wash out the syringe three or four times with this solution, or the contents of the syringe may be expelled into a tube containing 6 c.c. of warm normal salt solution and the syringe washed out with 3 c.c. fresh warm salt solution into this tube in order better to remove all trypanosomes from the syringe and needle. This gives a suspension of 1:100, and from this tube further suspensions of the trypanosomes may be made in warm, sterile salt solution as required.

The Dilution of Infected Blood for Transmissions.—A 1:100 suspension of infected blood having been secured, further suspensions may be made by transferring 1 c.c. to a series of test tubes containing 9 c.c. of warm, sterile salt solution. The 1:100 suspension and each succeeding one should be thoroughly mixed by furnishing each test tube with a sterile rubber stopper and then gently agitating the contents with turning up and down until a thorough admixture results. With the final suspensions, a series of rats may be infected by injection of each intraperitoneally with a cubic centimeter or fraction thereof, according to the count. An example will explain this step:

If a count of 84,000,000 trypanosomes (equiperdum) in a cubic centimeter of undiluted blood has been found, 0.1 c.c. blood + 0.9 c.c. citrate solution (No. 1) + 9 c.c. normal salt solution equals a 1:100 suspension (No. 2), each cubic centimeter of which contains 840,000 trypanosomes, or 0.1 c.c. contains 84,000 trypanosomes.

One c.c. of this suspension (No. 2) in 9 c.c. of normal salt solution gives a suspension (No. 3) of which 1 c.c. contains 84,000 trypanosomes. Rats may then be injected intraperitoneally with 1 c.c. of this suspension (84,000) — a procedure which is probably better than the injection of 0.1 c.c. of Suspension 2. Further dilutions may be made and fewer trypanosomes injected by using fractions of a cubic centimeter, as 0.5 c.c., in this instance containing 42,000 trypanosomes, or 0.2 c.c., containing 16,800, etc.

In conducting chemotherapeutic researches with T. equiperdum, best results have been obtained when the rats were infected with about 100,000 trypanosomes an animal. With T. brucei fewer may be used and with T. lewisi a larger number should be injected.

The Method of Infecting Rats.—Rats may now be infected by the intraperitoneal injection of the proper dose of trypanosome suspension. For this purpose, I prefer a 1 c.c. Record syringe fitted with a No. 22 needle. The suspension of trypanosomes should be briefly and gently mixed each time before the syringe is loaded in order to avoid settling. The syringe should be sterile and the technic fairly aseptic, altho I have never seen infection of the peritoneum follow when the rat was properly held, a touch of alcohol applied to the abdominal skin, and the needle properly entered. I have not found it necessary to clip the hair or sterilize the skin by washing or even by an application of tincture of iodin, and with due care the intestines are not punctured.

A NOTE ON THE NUMERIC RELATIONS OF TRYPANOSOMES TO INFECTION

Mainly for the purpose of testing the reliability of this method for transmitting trypanosomes, a number of experiments were conducted with T. lewisi, T. equiperdum, and T. brucei, consisting in the injection of a series of rats with increasing numbers for the purpose of determining three factors: (1) With how few trypanosomes of these three species rats may be infected; (2) the relation of the numbers of trypanosomes injected to the time of true incubation; (3) the relation of the number of trypanosomes injected to the age and duration of life of the infected animal.

Materials.—The three species of trypanosomes were obtained from the Rockefeller Institute through the kindness of Dr. Wade H. Brown. Trypanosoma lewisi at times developed a virulence for our albino rats, especially after a number of short interval transfers, an experience with this strain which is similar to that described by Jürgens[2] and Brown.[3]

Trypanosoma equiperdum, when injected in very large numbers, usually killed its host about the sixth to the tenth day and Trypanosoma brucei about the third to fifth day. These intervals were on several occasions considerably shortened by a series of short interval transfers.

The Method of Study.—The trypanosomes were counted after the method described and increasing numbers injected intraperitoneally into several series of rats, which were numbered and weighed. Daily examinations were made by the usual method of snipping the tip of the tail and directly examining a drop of blood on a cover glass and slide by means of a No. 6 objective and No. 4 eyepiece (Leitz). The number of trypanosomes were recorded after the method of + + + +, + + +, + +, +, and "few," already described. If no trypanosomes were found in at least ten consecutive fields, the result was recorded as negative. In all instances, large, freely flowing drops of blood were examined; blood obtained by undue squeezing and milking of the tail, as stated before, is unreliable.

The results of some of the experiments are shown in the following groups of tables with a summary accompanying each group.

TRYPANOSOMA LEWISI (TABLES 2, 3, 4, AND 5)

(1) The smallest number of T. lewisi found capable of infecting a rat was 138 (Table 5); the animal was full grown and weighed 138 grams. The same number injected into a rat of lighter weight failed to infect. The next lowest number was 190, in a rat weighing 46 gm. (Table 2).

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[2] Arch. f. Hyg., 1902, 42, p. 265.

[3] Jour. Exper. Med., 1914, 19, 406; ibid., 1915, 21, p. 345.

TABLE 2

Trypanosoma Lewisi *

No. of Rat	Weight, in Grams	Number of Trypanosomes Injected Intra-peritoneally	Results of Examination of Blood from Tail										
			24 hr.	36 hr.	48 hr.	72 hr.	96 hr.	5 da.	6 da.	7 da.	8 da.	9 da.	10 da.
1	52	95	—	—	—	—	—	—	—	—	—	—	—
2	46	190	—	—	—	—	—	—	—	+	++	++++	Died
3	50	420	—	—	—	—	—	—	Died	0	0	0	0
4	53	1,680	—	—	—	—	Few	Few	++	++++	++++	Died	0
5	109	4,200	—	—	—	—	—	—	—	++	+++	++++	Died
6	136	8,400	—	—	—	—	—	—	—	++	+++	++++	Died
7	115	42,000	—	—	—	—	Few	+	++++	++++	Died	0	0
8	125	84,000	—	—	—	—	+	++	++++	++++	++++	Died	0

* Seed rat showed + + + +: 42 trypanosomes in 400 small squares (dilution 1: 200) = 84.000,000 trypanosomes in 1 cubic millimeter undiluted blood, and 84,000,000 in 1 cubic centimeter.

TABLE 3

Trypanosoma Lewisi *

No. of Rat	Weight in Grams	Number of Trypanosomes Injected	Results of Examination of Blood from Tail															
			24 hr.	48 hr.	72 hr.	96 hr.	5 da.	6 da.	7 da.	8 da.	9 da.	10 da.	11 da.	12 da.	13 da.	14 da.	15 da.	16 da.
1	67	186	—	—	—	—	—	—	—	—	—	+	+++	++++	++++	++++	++++	++++
2	73	1,116	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
3	62	7,440	—	—	—	—	Few	+	++	++	+++	Died						
4	60	14,880	—	—	—	—	Few	++	+++	+++	+++	++++	++++	++++	Died			
5	62	18,600	—	—	—	—	Few	++	+++	++++	Died							
6	54	93,000	—	—	—	—	+	+++	+++	++++	Died							
7	73	186,000	—	—	—	—	—	—	—	—	—	—	—	+	+++	++++	++++	++++

* Seed rat showed + +: 93 trypanosomes in 400 small squares (dilution 1: 20)=18,600 trypanosomes in 1 cubic millimeter undiluted blood, and 18,600,000 in 1 cubic centimeter.

TABLE 4

TRYPANOSOMA LEWISI *

No. of Rat	Weight in Grams	Number of Trypanosomes Injected	Results of Examination of Blood from Tail											
			24 hr.	48 hr.	72 hr.	96 hr.	5 da.	6 da.	7 da.	8 da.	9 da.	10 da.	11 da.	12 da.
1	56	300	—	—	—	—	—	Few	+	+++	+++	++++	++++	++++
2	70	1,500	—	—	—	—	Few	++	+++	++++	++++	++++	++++	++++
3	82	3,000	—	—	—	—	—	Few	+	++	++	++++	++++	++++
4	91	15,000	—	—	—	—	—	+	++	++++	Died	0	0	0
5	102	30,000	—	—	—	—	—	—	—	—	—	—	—	+
6	83	60,000	—	—	—	—	—	—	—	—	Died	0	0	0
7	96	150,000	—	Died	0	0	0	0	0	0	0	0	0	0
8	72	3,000,000	—	—	—	—	—	+	++	+++	++++	++++	++++	++++

* Seed rat showed + + +: 15 trypanosomes in 400 small squares (dilution 1: 200)=30,000 trypanosomes in 1 cubic millimeter undiluted blood, and 30,000,000 in 1 cubic centimeter.

TABLE 5

TRYPANOSOMA LEWISI *

No. of Rat	Weight in Grams	Number of Trypanosomes Injected	Results of Examination of Blood from Tail														
			24 hr.	48 hr.	72 hr.	96 hr.	5 da.	6 da.	7 da.	8 da.	9 da.	10 da.	11 da.	12 da.	13 da.	14 da.	15 da.
1	97	138	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
2	138	138	—	—	—	—	—	—	—	Few	+	++	++	—	—	—	—
3	128	690	—	—	—	Few	Few	Few	+	++++	++++	++++	++++	++++	++++	++++	++++
4	83	1,380	—	—	—	Few	Few	+	++	++++	++++	++++	++++	++++	++++	++++	++++
5	120	13,800	—	—	—	Few	Few	++	+++	++++	++++	++++	++++	++++	++++	++++	++++

* Seed rat showed + +: 69 trypanosomes in 400 small squares (dilution 1:20)=13,800 trypanosomes in 1 cubic millimeter undiluted blood, and 13,800,000 in 1 cubic centimeter.

Trypanosoma lewisi under ordinary conditions is to be regarded as feebly aggressive and pathogenic for the majority of albino rats, so that, as a rule, more of these trypanosomes are required to induce uniform infection in fresh animals than are required of T. equiperdum and T. brucei. Sometimes relatively large numbers failed to infect normal animals, an example being found in Table 4, Rat 6, weighing 83 gm., which the injection of 60,000 trypanosomes did not infect to the extent that the trypanosomes could be found in the peripheral blood up to the ninth day, when it died. In our experience, at least 100,000 trypanosomes should be injected intraperitoneally in order to secure uniform infections.

(2) The time of true incubation of Trypanosoma lewisi did not bear any constant or striking relation to the number of trypanosomes injected. In general, the period of incubation was found to be about five days. As will be noted in Table 2, the injection of 1,680 trypanosomes into the peritoneal cavity of a rat weighing 53 gm. was followed by a four day period of incubation, the same interval following the injection of 84,000 trypanosomes into a rat of 125 gm. weight; it must be noted that the second rat was more than twice as heavy as the first. But a similar result is noted in Table 3; in this example an injection of 7,440 trypanosomes into a rat weighing 62 gm. was followed by the same five day incubation period as followed the injection of 93,000 trypanosomes into a rat weighing 54 gm.; in this same series, the injection of 186,000 trypanosomes into a rat of 73 gm. weight (Rat 7) was followed by a twelve day incubation period. Similar examples are found in Tables 4 and 5.

It is to be remembered however that the largest of these doses did not contain nearly as many trypanosomes as are injected by the ordinary technic, especially when undiluted, defibrinated blood is used. With such technic, the time of true incubation is about three or four days and, indeed, adult forms of the trypanosomes may be found in the blood within twenty-four hours, an example of the so-called "false" incubation.

(3) With regard to the relation of the size of the infecting dose to the weight of the animal and to the period of incubation, this series was too short to warrant a conclusion, but, in general, it may be stated that the younger the rat the more subject it is to infection.

Individual resistance of the rat influenced Trypanosoma lewisi to a far greater extent than was evident with T. equiperdum and especially T. brucei.

TABLE 6

Trypanosoma Equiperdum *

No. of Rat	Weight in Grams	Number of Trypanosomes Injected	Results of Examination of Blood from Tail											
			24 hr.	48 hr.	72 hr.	96 hr.	5 da.	6 da.	7 da.	8 da.	9 da.	10 da.	11 da.	12 da.
1	76	6	—	—	—	—	—	—	—	—	—	—	—	—
2	56	36	—	—	—	—	—	—	—	—	—	—	—	—
3	71	72	—	—	—	—	—	—	—	Died	0	0	0	0
4	59	288	—	—	—	—	—	—	—	—	—	—	—	—
5	73	243	—	—	—	—	—	—	Few	+	+	++	+++	+++
6	108	486	—	—	—	—	Few	++	++++	++++	Died	0	0	0
7	109	2,430	—	—	—	—	+	++	++++	++++	++++	++++	++++	Died
8	98	4,860	—	—	—	—	—	+	++	++++	++++	++++	++++	++++
9	115	24,300	—	—	—	Few	++++	++++	++++	Died	0	0	0	0
10	112	48,600	—	—	+	+++	++++	++++	++++	++++	++++	++++	Died	0
11	117	243,000	—	—	+	+++	++++	Died	0	0	0	0	0	0
12	134	486,000	—	—	—	—	+	++	++++	++++	++++	++++	++++	Died

* Seed rat showed + + + +: 243 trypanosomes in 400 small squares (dilution 1: 200) = 486,000 trypanosomes in 1 cubic millimeter undiluted blood, and 486,000,000 in 1 cubic centimeter.

TABLE 7

Trypanosoma Equiperdum *

No. of Rat	Weight in Grams	Number of Trypanosomes Injected	Results of Examination of Blood from Tail								
			24 hr.	48 hr.	72 hr.	96 hr.	5 da.	6 da.	7 da.	8 da.	9 da.
1	74	2,400	—	—	—	Few	++	++++	++++	++++	Died
2	75	4,800	—	—	—	Few	+++	++++	++++	++++	++++
3	70	14,400	—	—	Few	++	+++	++++	Died	0	0
4	65	24,000	—	—	+	++	Died	0	0	0	0
5	72	38,400	—	—	+	+++	++++	++++	++++	++++	Died
6	66	48,000	—	—	++	++++	++++	++++	++++	Died	0
7	66	144,000	—	Few	+++	++++	++++	++++	++++	++++	Died
8	70	240,000	—	Few	++++	++++	++++	++++	++++	++++	Died
9	95	384,000	—	Few	++++	++++	++++	++++	++++	++++	Died
10	60	480,000	—	—	++++	Died	0	0	0	0	0

* Seed rat showed + +: 23 trypanosomes in 400 small squares (dilution 1: 200) = 4,800 trypanosomes in 1 cubic millimeter undiluted blood, and 4,800,000 in 1 cubic centimeter.

The death rate among rats infected with this strain of Trypanosoma lewisi was variable, without particular relation to the weight of the animal or the number of trypanosomes injected. Most of these experiments were conducted at a time when the strain had an exalted virulence, so that many of the animals succumbed within twenty days after infection.

TRYPANOSOMA EQUIPERDUM (TABLES 6 AND 7)

(1) The smallest number of T. equiperdum found to infect a rat was 243 in an animal weighing 73 gm. (Rat 5, Table 6), trypanosomes being found in the blood on the seventh day. Probably a more extensive series of experiments would discover rats that could be infected with a smaller number, as was found with Trypanosoma lewisi. In our chemotherapeutic work, we regularly inject our rats with from 80,000 to 100,000 of these trypanosomes in order to insure uniform infections.

(2) Some relation was found between the number of trypanosomes injected and the time of true incubation. As noted in Table 7, the injection of 144,000 and more trypanosomes was followed by a forty-eight-hour period of incubation; the injection of 15,000 to 48,000 was followed by a seventy-two-hour incubation, and the injection of smaller numbers, as 2,400-4,800 and 500-4,860, was followed by periods of four and five days, respectively, before trypanosomes could be found in the peripheral blood.

(3) There was found no constant relation between the number of trypanosomes injected and the duration of life under the conditions of these experiments. While the period of incubation was lengthened by injecting a small number of trypanosomes, the duration of life was in general the same, after trypanosomes were found in the peripheral blood, as when larger numbers were injected. Occasionally rats died on the third day after trypanosomes were found in the blood, but usually the time of death varied from the fifth to the eighth day.

TRYPANOSOMA BRUCEI (TABLES 8, 9, AND 10)

(1) In our experiments, the smallest number of T. brucei found capable of infecting a rat was 37 in an animal weighing 95 gm., the parasites appearing in the blood after an eight day period of incubation (Rat 1, Table 8). The injection of as few as 150 of these trypanosomes infected rats weighing 84 and 76 gm. respectively, in two different experiments (Rat 1, Table 9 and Rat 5, Table 10).

TABLE 8

TRYPANOSOMA BRUCEI *

No. of Rat	Weight in Grams	Number of Trypanosomes Injected	Results of Examination of Blood from Tail											
			24 hr.	48 hr.	72 hr.	96 hr.	5 da.	6 da.	7 da.	8 da.	9 da.	10 da.	11 da.	12 da.
1	95	37	—	—	—	—	—	—	—	—	+	++	+++	Died
2	106	74	—	—	—	—	—	—	—	—	—	Died	0	0
3	107	370	—	—	—	—	—	—	—	—	—	—	—	—
4	117	740	—	—	Few	+++	++++	Died	0	0	0	0	0	0
5	113	1,480	—	—	++	++++	Died	0	0	0	0	0	0	0
6	78	4,440	—	++	++	++++	Died	0	0	0	0	0	0	0
7	69	7,400	—	++	+++	++++	++++	++++	Died	0	0	0	0	0
8	45	14,800	—	—	Few	+	++++	++++	Died	0	0	0	0	0

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* Seed rat showed + + +: 37 trypanosomes in 400 small squares (dilution 1: 200) = 74,000 trypanosomes in 1 cubic millimeter undiluted blood, and 74,000,000 in 1 cubic centimeter.

Trypanosoma brucei was found the most aggressive and virulent of the three species of trypanosomes studied, the injection of small numbers being followed most regularly by uniform infections.

(2) A relation was found between the number of trypanosomes injected and the period of incubation.

In general, this relation was found most constant with these trypanosomes, somewhat so with Trypanosoma equiperdum, and least with Trypanosoma lewisi, having in this manner some bearing on the aggressiveness of these various parasites for the rat.

TABLE 9

TRYPANOSOMA BRUCEI *

No. of Rat	Weight in Grams	Number of Trypanosomes Injected	Results of Examination of Blood from Tail						
			24 hr.	48 hr.	72 hr.	96 hr.	5 da.	6 da.	7 da.
1	84	150	—	—	—	—	++	++++	Died
2	93	180	—	—	—	+++	++++	++++	Died
3	88	600	—	—	Few	++	++++	++++	Died
4	72	1,500	—	—	Few	+++	++++	++++	Died
5	66	3,000	—	—	+	++++	++++	++++	Died
6	98	9,000	—	Few	+	++++	Died	0	0
7	70	15,000	—	+	++	++++	++++	++++	Died
8	76	30,000	—	—	++	++++	++++	++++	Died

* Seed rat showed + + +: 15 trypanosomes in 400 small squares (dilution 1: 200) = 30,000 trypanosomes in 1 cubic millimeter undiluted blood, and 30,000,000 in 1 cubic centimeter.

TABLE 10

TRYPANOSOMA BRUCEI *

No. of Rat	Weight in Grams	Number of Trypanosomes Injected	Results of Examination of Blood from Tail						
			24 hr.	48 hr.	72 hr.	96 hr.	5 da.	6 da.	7 da.
1	70	2	—	—	—	—	—	—	—
2	78	8	—	—	—	—	—	—	—
3	79	40	—	—	—	—	—	—	—
4	98	80	—	—	—	—	—	—	—
5	76	149	—	—	—	Few	+	++++	Died
6	92	298	—	—	—	+	++++	++++	Died
7	80	1,490	—	—	Few	++	++++	Died	0
8	85	2,980	—	—	+	++++	++++	++++	Died
9	91	14,900	—	—	++	++++	Died	0	0
10	77	29,800	—	Few	+++	++++	++++	++++	Died
11	110	149,000	—	+	++++	Died	0	0	0
12	82	298,000	—	+	++	++++	++++	++++	Died

* Seed rat showed + + +: 149 trypanosomes in 400 small squares (dilution 1: 20) = 29,800 trypanosomes in 1 cubic millimeter undiluted blood, and 29,800,000 in 1 cubic centimeter.

After injecting very large numbers of T. brucei, as contained, for example, in 0.2-0.3 c.c. of undiluted, defibrinated blood, I have on occasion found trypanosomes in the peripheral blood in twenty-four hours.

The intraperitoneal injection of about 5,000 (Rat 6, Table 8) to about 300,000 (Rat 12, Table 10) trypanosomes was followed by a period of incubation of about forty-eight hours; possibly prolonged examinations at more frequent intervals would have shown an earlier appearance of the parasites in the peripheral blood with the larger doses. The injection of about 600 (Rat 3, Table 9) to 3,000 (Rats 4 and 5, Table 8 and Rats 3, 4, and 5, Table 9) trypanosomes was followed by a seventy-two-hour period of incubation, and the injection of 150 to 300 trypanosomes (Tables 9 and 10) by a four day interval.

(3) Ordinarily, rats heavily injected with this strain of Trypanosoma brucei died within three or four days after the intraperitoneal injection of parasites. Infection with smaller numbers lengthened the duration of life; but after the parasites had multiplied to such an extent as to be found in the peripheral blood, the duration of life was about uniform (three to five days), regardless of how many trypanosomes had been injected at the time of infection. In this manner, there was a distinct numeric relation between the number of trypanosomes injected and the time of death in so far that the fewer injected the longer the time required for sufficient multiplication of numbers to fatally intoxicate the host.

CONCLUSIONS

A method is described which has been found simple, fairly accurate, and very useful for transmitting approximately known numbers of trypanosomes to normal rats under sterile conditions and without injury to the seed animal.

By this method, several series of rats have been infected with increasing numbers of Trypanosoma lewisi, T. equiperdum, and T. brucei, and a study made of the minimal number of trypanosomes in each species necessary for infection, as well as the time of true incubation and duration of life. Well-defined quantitative relations were found with T. brucei, less with T. equiperdum, and least with T. lewisi.

The injection of small numbers of pathogenic trypanosomes lengthens the period of incubation and, in this manner, the duration of life, as dating from the time of infection; but when the parasites once appear in the peripheral blood, the duration of life is about the same, regardless of whether the animal was originally infected with a large or a small number.